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Honors Thesis:

Differential expression of *Elizabethkingia anophelis* to cefotaxime and imipenemAbstract

Elizabethkingia anophelis is a beta-lactam antibiotic resistant bacterium that uses a variety of mechanisms to express this resistance. It is currently unknown how *E. anophelis* responds when exposed to different beta-lactam antibiotics and what genes will up or down regulate different genes in order to express antibiotic resistance. When exposed to cefotaxime it appears that *E. anophelis* will up regulate the production of 4 putative RND efflux pumps along with slight up regulation of a single putative peptidoglycan synthesis protein. This is done in conjunction with a relevant increase fold change in the beta-lactamase BlaB by a factor of +1.5 and an average decreases of -2.33 in the production of GOB, CME, and putative beta-lactamases TLA and Open Reading frame 666. However, when exposed to imipenem *E. anophelis* will instead down regulate the production of RND efflux pumps, and slightly up regulate the production of a single peptidoglycan synthesis protein. This combined with a decrease in production of BlaB and CME and a relevant increase GOB and ORF666. Both GOB and ORF666 increased by an average fold change of +2.1 with a mRNA count increase of +1,552 leads to a unique response. This difference in the change in the expression of beta-lactamases, peptidoglycan synthesis and efflux pumps under cefotaxime and imipenem conditions shows different responses within a cell to when reaction with different beta-lactam antibiotics. Finally,

the observed response by ORF666 suggests that this putative beta-lactamase may play an important role in the resistance of *E. anophelis* to imipenem, and future work is needed to establish if this is a functional beta-lactamase.

Introduction

Elizabethkingia anophelis is an important emerging opportunistic human pathogen with mortality rate of around 25%,¹ and is known to cause meningitis, bloodstream, and respiratory infections in young children and immunocompromised individuals.¹ Infections caused by *E. anophelis* are also notable because they can be passed from an infected mother to a newborn child and can cause neonatal meningitis.^{2,3} *Elizabethkingia anophelis* has a low rate of infection and typically infects around 5-10 individuals in the United States per year.⁴ However, outbreaks have occurred around the world in places such as Wisconsin, Michigan, Illinois, China, and the Central African Republic.^{3,5} In the midwestern US outbreak (2016) 63 individuals were infected by *E. anophelis* and 18 of those patients died (28.5% mortality).⁴ This high mortality rate of *E. anophelis* is due to combination of antibiotic resistance to multiple drugs lack of knowledge of the species, and the immunocompromised status of infected individuals. These factors combined have led to ineffective treatment options and have caused *E. anophelis* to be clinically relevant for research.

In order to obtain a further understanding we will be focusing on the identifying the possible mechanisms of antibiotic resistance to beta-lactam antibiotics. Under normal conditions beta-lactam antibiotics work to disrupt and destroy the synthesis of the cell wall of bacteria.⁶ This is done by inhibiting cell wall synthesis proteins, known as penicillin binding proteins (PBP). Inhibition of these enzymes causes the cell to be unable to replace and repair the peptidoglycan cell wall and causes eventual lysis of the cell.⁷ However, multiple bacteria such as

Elizabethkingia anophelis have developed mechanisms of resistance.³ One such mechanism is the creation of beta-lactamases, which function by hydrolyzing the lactam ring on beta-lactam antibiotics. This hydrolysis causes the beta-lactam antibiotic to become unable to bind and inhibit penicillin-binding proteins. Another mechanism for beta-lactam resistance is caused from the production of multiple variations of penicillin binding proteins (PBP). This variation in penicillin binding proteins can be characterized in Methicillin-resistant *Staphylococcus aureus*⁸ and *Actinobacteria* species.⁹ These variations cause antibiotic resistance by significantly lowering the binding affinity of beta-lactams to the new penicillin binding proteins.^{8,9} This causes beta-lactams to be ineffective at inhibiting the penicillin binding proteins making treatment ineffective. There are currently no classified novel penicillin binding proteins identified in *E. anophelis*, but it is still something to consider when examining putative PBP.

Another form of antibiotic resistance is the expression of efflux pumps. Efflux pumps function to transport foreign material and cellular components outside of the cytoplasm or outside the cell. This allows for the excretion of beta-lactam antibiotics from the periplasmic space.¹⁰ Li *et al.* (1994) showed that *Pseudomonas aeruginosa* could remove hydrophilic beta-lactam antibiotics by measuring the cellular concentration of the hydrophilic antibiotic azlocilin at 20 minute time intervals. The concentration was measured by using dry cell mass to determine the molarity of the antibiotic in the cell. They found that over time the concentration in the cell decreased at a steady rate even in strains with low beta-lactam production.¹⁰ Li *et al.* later performed a similar experiment on a low beta-lactamase producing and a drug-hypersusceptible mutant known as K799/61. This experiment followed the same procedure of the original experiment and had similar results, which helped to further confirm their original findings by demonstrating that cells lacking beta-lactamases could provide some form of antibiotic resistant mechanism by increasing

the expression of efflux pumps.¹¹ Pages *et al.* (2009) also showed that the introduction of efflux pumps into *Klebsiella pneumoniae* caused increased antibiotic resistance to cefoxitin. This was done by using a *Klebsiella pneumoniae* strain that had a genome lacking the two characterized beta-lactamases of TEM- and AmpC-. After confirming the absence of these beta-lactamases they performed an immunodetection analysis of efflux pumps in the cell grown under antibiotic conditions. This immunodetection found that there were multiple efflux pumps being produced by the cells and that they existed in higher quantities compared to normal conditions. According to pages, this finding suggests that efflux pumps cause these antibiotic susceptible strain to be antibiotic resistant.

Elizabethkingia anopheles expresses three characterized beta-lactamases known as GOB, CME1, and BlaB.^{15, 16} However, besides from these beta-lactamases there is very little information known about *Elizabethkingia anophelis* and the regulation of gene expression under antibiotic pressures is not understood. It has been predicted by whole genome sequencing that *Elizabethkingia anophelis* carries 19 β -lactamases, including 16 putative, four metallo- β -lactamases (MBLs), two of which are putative, four putative penicillin-binding proteins and 13 putative RND efflux pump proteins^{13,14}. In this thesis, I will be examining all three of these pathways in order to investigate changes in expression of beta-lactamases, peptidoglycan synthesis and efflux pumps. I hypothesize that the expression of genes in the related three pathways will differ between exposures to beta-lactam antibiotics. In order to test the change in gene expression we examined the change in mRNA counts in *Elizabethkingia anophelis* under standard growth conditions and compared the mRNA counts under exposure to imipenem and cefotaxime. The three mechanisms mentioned above were specifically chosen because they are

potentially involved in antibiotic resistance. We found that there was a change in expression among cefotaxime and imipenem in all pathways.

Methods

In order to test this hypothesis *Elizabethkingia anophelis* R26 was grown in three separate 20mL cultures. A single colony was inoculated into 20mL of Mueller-Hinton broth (MHB) and grown over night at 37°C with shaking at 200rpm. After being allowed to grow the cultures were then diluted to an optical density at 600nm (OD₆₀₀) of 0.1 and allowed to grow at 37°C to mid exponential phase (OD₆₀₀ = 0.8). They were then exposed to the relevant antibiotic for 20 minutes. The first culture contained no antibiotics and served as the negative control for the experiment. The second culture contained MHB and had 2ug/ml of imipenem added. Finally the last culture contained MHB media and had 4ug/ml cefotaxime added. After the cultures were grown the total RNA was extracted and purified. After purification the RNA was then shipped to the Macrogen Company (Rockvill, MD) for pair end sequencing. After receiving the paired end RNA sequences they were placed into the Rockhopper program in order to pair together the RNA sequences.^{18,19} This helped to determine the total numbers of genes present in the genome as well as the total number of mRNA counts for each gene. The total mRNA count for the control sample was then compared to the antibiotic grown *E. anophelis* in order to show the fold change in transcription. We determined that any gene that had over 30 total mRNA counts between the three cultures and had a fold change of 1.5 or higher were relevant changes. Once this information was determined the bacterial genome was entered into the KEGG automated annotation program in order to show the gene locations in the pathways of *Elizabethkingia anopholis*.²⁰ This information was then used to show the fold change of genes

within the peptidoglycan biosynthesis, efflux pump and the beta-lactamase pathway under antibiotic conditions.

Results

Elizabethkingia anophelis produce 15 proteins that are involved in the peptidoglycan biosynthesis pathway. All of these proteins are considered putative because they have been identified only through sequence identification. Regardless, these 14 putative genes were found at open reading frames (ORF) 187, 275, 269, 644, 646, 928, 995, 1250, 2285, 2284, 2287, 2288, and 3373. The putative enzyme name, fold change, and operons for each ORF can be found in table 1. These genes were found to span nearly the entire pathway (Figure 1). The fold change for all proteins can be found in figure 2. Out of the 14 putative genes there were 5 genes (ORF. 187, 646, 995, 1250, and 3396) that encoded for the penicillin binding protein D-alanyl-D-alanine carboxypeptidase¹⁷ in the pathway and are highlighted in yellow in table 1. D-alanyl-D-alanine carboxypeptidase can be found at ORFs 187, 646, 995, 1250, and 3386. Under cefotaxime conditions, ORF 1250 was the only gene to have a relevant positive fold change (+1.6). This fold change lead to a +286 mRNA count increase compared to control conditions. There was also relevant fold change decrease at ORFs 187 and 646 (-1.6 and -2.6) following exposure to cefotaxime. The fold change decrease at ORFs 187 & 646 lead to a total decrease of -121 counts. Both the increase and decrease in mRNA count lead to an overall mRNA count increase of +165 for the putative D-alanyl-D-alanine carboxypeptidase genes.

Under imipenem conditions there was only one relevant positive fold change at ORF 995 and no relevant negative fold changes. This change at ORF 995 changed the mRNA count from 10 to 17 and caused a total mRNA count increase of +7.

Table 1: mRNA fold change for peptidoglycan synthesis pathway with operons. mRNA counts too low for relevance are in blue, fold change increases are in green, and fold change decreases are in orange. Relevant proteins are in yellow

ORF	Gene Description	mRNA Count Control	mRNA Counts Cefotax	mRNA Counts Imipenem	Fold Change Cefotax	Fold Change Imipenem	Operonic?
187	D-alanyl-D-alanine carboxypeptidase (EC 3.4.16.4)	294	189	350	-1.6	1.2	187, 188
269	UDP-N-acetylglucosamine 1-carboxyvinyltransferase (EC 2.5.1.7)	29	13	21	-2.2	-1.4	268, 269
275	UDP-N-acetylenolpyruvoylglucosamine reductase (EC 1.1.1.158)	72	36	71	-2.0	-1	None
644	Prolipoprotein diacylglycerol transferase (EC 2.4.99.-)	69	32	64	-2.2	-1.1	644, 645, 646
646	D-alanyl-D-alanine carboxypeptidase (EC 3.4.16.4)	34	13	41	-2.6	1.2	644, 645, 646
928	UDP-N-acetylmuramoylalanyl-D-glutamyl-2,6-diaminopimelate--D-alanyl-D-alanine ligase (EC 6.3.2.10)	570	748	513	1.3	-1.1	None
995	D-alanyl-D-alanine carboxypeptidase (EC 3.4.16.4)	10	6	17	-1.7	1.7	994, 995
1250	D-alanyl-D-alanine carboxypeptidase (EC 3.4.16.4)	463	749	373	1.6	-1.2	1250, 1251
2285	UDP-N-acetylmuramoylalanyl-D-glutamate ligase (EC 6.3.2.9)	69	53	132	-1.3	1.9	2284, 2285, 2286, 2287, 2288, 2289
2284	Phospho-N-acetylmuramoyl-pentapeptide-transferase (EC 2.7.8.13)	76	39	62	-1.9	-1.2	2284, 2285, 2286, 2287, 2288, 2289
2283	UDP-N-acetylmuramoylalanyl- D-glutamate : 2,6-diaminopimelate ligase	369	366	293	-1	-1.3	2280, 2281, 2282, 2283
2287	UDP-N-acetylglucosamine--N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase (EC 2.4.1.227)	1908	2971	1574	1.6	-1.2	2284, 2285, 2286, 2287, 2288, 2289
2288	UDP-N-acetylmuramate--alanine ligase (EC 6.3.2.8)	5903	8416	3858	1.4	-1.5	2284, 2285, 2286, 2287, 2288, 2289
2954	Undecaprenyl-diphosphatase (EC 3.6.1.27)	6	6	10	2.5	3.5	2952, 2953, 2954, 2955, 2956
3373	D-alanine--D-alanine ligase (EC 6.3.2.4)	29	17	40	-1.7	1.4	3373, 3374, 3375
3396	D-alanyl-D-alanine carboxypeptidase (EC 3.4.16.4)	21	23	16	1.1	-1.3	None

Peptidoglycan

Biosynthesis pathway

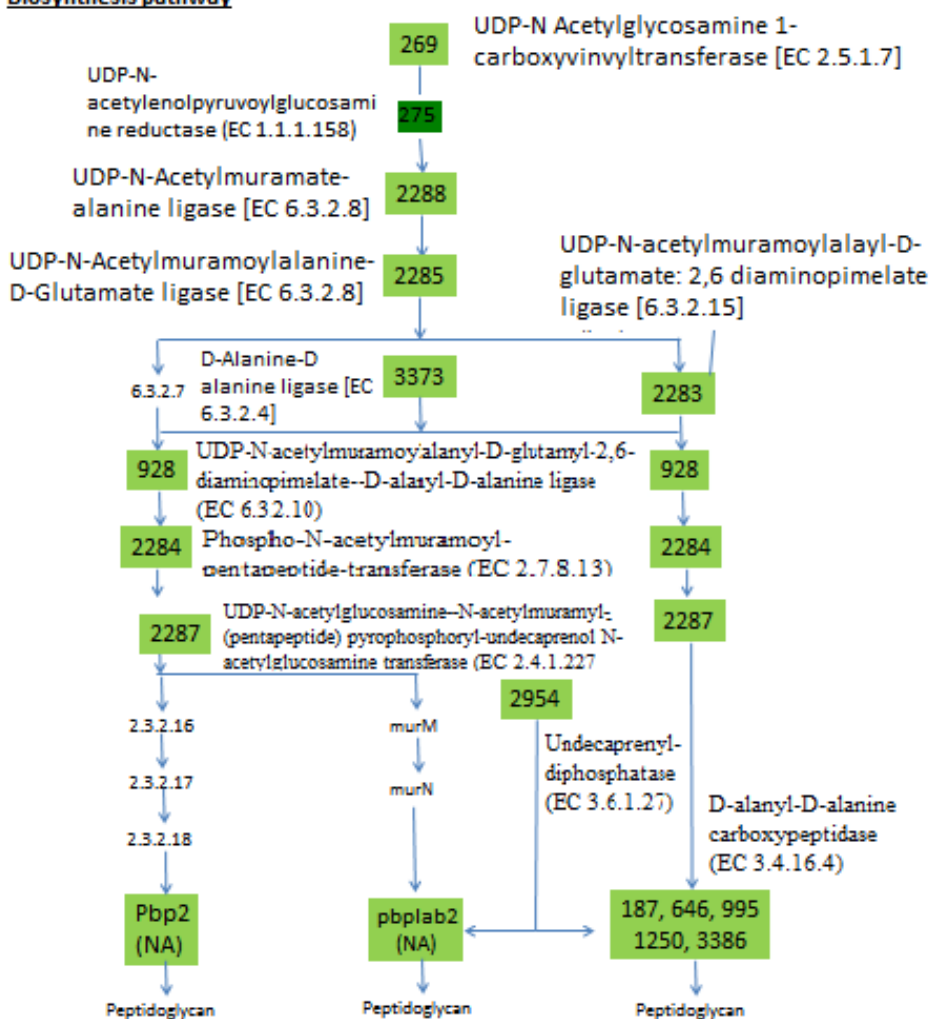


Figure 1: Peptidoglycan synthesis pathway proteins recognized by KEGG are highlighted green with ORF in boxes. Boxes with enzyme code were not found in gene list. Enzymes marked with NA were recognized by KEGG but not found in gene list. Name of gene next to the ORF box.

Adapted from: [http://www.genome.jp/kegg-](http://www.genome.jp/kegg-bin/show_pathway?org_name=eao&mapno=00550&mapscale=&show_description=hide)

[bin/show_pathway?org_name=eao&mapno=00550&mapscale=&show_description=hide](http://www.genome.jp/kegg-bin/show_pathway?org_name=eao&mapno=00550&mapscale=&show_description=hide)

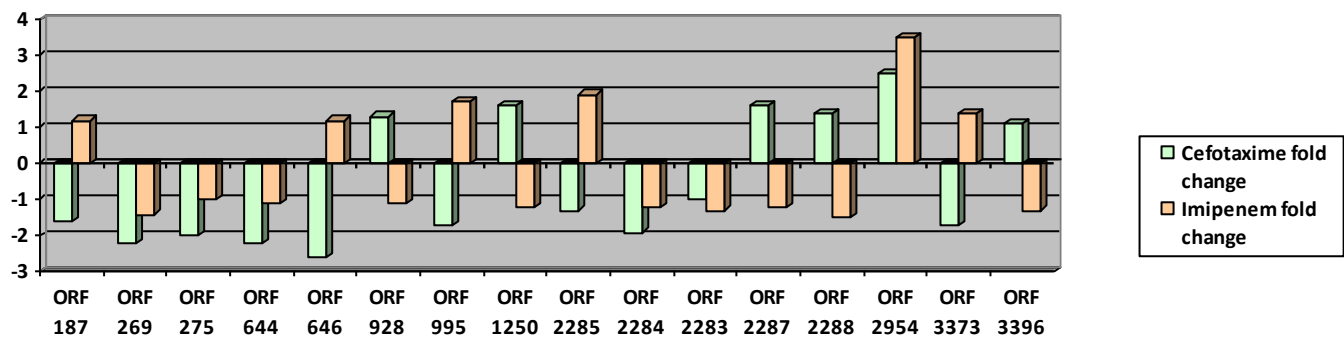


Figure 2: Fold change graph for peptidoglycan synthesis pathway proteins.

Within the KEGG antibiotic resistance pathway there are seven proteins that produced putative RND efflux pumps. All seven of these proteins were located at the same position in this pathway and are labeled as RND efflux pumps. The genes that encode for putative RND efflux pumps at ORFs 811, 1584, 1821, 2867, 2868, 2869, and 3436 (Table 2). Under cefotaxime conditions there were relevant fold change increases in ORFs 1821, 2868, 2869, and 3436 and no relevant fold change decreases when compared to the control. ORF 1821 encodes for a putative RND multidrug efflux transporter Acriflavin resistance protein. The putative multi-drug transporter we observed a fold change increase of +1.6 and changed the mRNA count from 9 to 14 (+5). ORF 2868 encoded for a putative RND efflux system, inner membrane transporter *cmeB*. For the putative *cmeB* transport system we observed a fold change increase of +1.6 and changed the mRNA count from 98 to 156 (+58). ORF 2869 encoded for a putative efflux transporter, RND family, MFP subunit. For the putative subunit we observed a fold change increase of +4.4 and changed the mRNA count from 1299 to 2067 (+768). Finally, ORF 3436 encoded for a putative heavy metal RND efflux outer membrane protein, *czcC* family. For the putative *czcC* outer membrane protein we observed a fold change increase of +1.8 and had an mRNA count change from 207 to 910 (+703). All 4 of these genes combined lead to a total relevant mRNA increase of +1534.

Under imipenem conditions, there was a relevant fold change increase in ORFs 811 and 1821. ORF 2867 also had a fold change increase greater than 1.5 levels, but had mRNA counts

were too low to be considered relevant. For ORF 811 (putative *cmeB* transporter) we observed a fold change increase of +1.8 and changed the mRNA count from 9 to 16 (+7). For ORF 1821 (putative multi-drug pump) we observed an increased fold change of +1.6 and changed the mRNA count from 9 to 14 (+5). There was also relevant fold decrease at ORFs 2869. For ORF 2869 (putative MFP subunit) we observed a fold change decrease of -1.6 and changed the mRNA count from 1299 to 800 (-499). When examining the mRNA change of the up and down regulated genes it can be seen that ORF 2869 had a total mRNA count drop by -499. While the increases from ORFs 811 and 1821 only had a total mRNA increase of +13. This leads to an overall decrease in mRNA count of -486 (Figure 3).

Table 2: mRNA fold change chart for RND efflux pumps production with operons. mRNA counts too low for relevance are in blue, fold change increases are in green, and fold change decreases are in orange

ORF	Gene Description	mRNA Count Control	mRNA Counts Cefotax	mRNA Counts Imipenem	Fold Change Cefotax	Fold Change Imipenem	Operonic?
811	RND efflux system, inner membrane transporter (CmeB)	9	13	16	1.4	1.8	808, 809, 810, 811
1584	efflux transporter, RND family, MFP subunit	726	1025	590	1.4	-1.2	1584, 1585, 1586
1821	RND multidrug efflux transporter; Acriflavin resistance protein	9	14	14	1.6	1.6	1820, 1821
2867	RND efflux system, outer membrane lipoprotein, NodT family	3	6	10	2	3.3	2867, 2868, 2869
2868	RND efflux system, inner membrane transporter CmeB	98	156	93	1.6	-1.1	2867, 2868, 2869
2869	efflux transporter, RND family, MFP subunit	1299	2067	800	1.6	-1.6	2867, 2868, 2869
3436	Heavy metal RND efflux outer membrane protein, CzcC family	207	910	301	4.4	1.4	3434, 3435, 3436

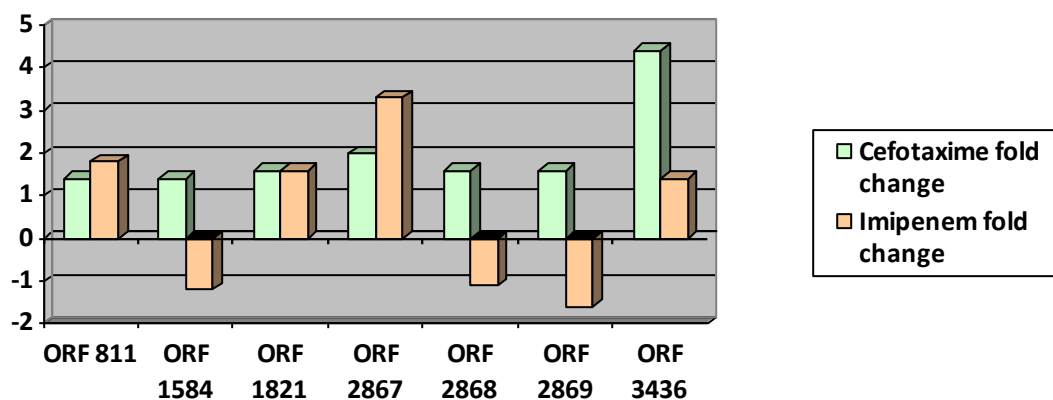


Figure 3: Fold change graph RND efflux pumps.

In addition, 15 proteins produced putative beta-lactamase products. These proteins are found at ORFs 29, 194, 339, 503, 519, 605, 666, 1010, 1103, 1724, 1915, 1990, 2536, 2959, and 3527. All of these proteins are considered putative except for those located at ORF 503, 1915, and 2054, which encode for *CME*, *blaB* and *GOB* respectively. Under cefotaxime conditions there were relevant positive fold changes at ORF 605, 1103 and 1915 (*blaB*). For ORF 605 we observed a fold change increase of 1.7 and changes the mRNA count from 471 to 797 (+320). For ORF 1103 we observed a fold change increase of +1.7 and changes mRNA count from 63 to 106 (+43). For *BlaB* (ORF 1915) we observed a fold change increase of +1.5 and changed from 89 to 144 (+55). There were also relevant fold change decreases in ORFs 519, 666, 1010 (putative *TLA*) and 2054 (*GOB*). For ORF 519 we observed a negative fold change of -2.5 and changed the mRNA count from 48 to 19 (-29). For Orf 666 we observed a fold change decrease of -3.8 and changed the mRNA count from 930 to 242 (-688) while the putative *TLA* (1010) we observed a fold change decrease of -2.1 and changed mRNA count from 71 to 34 (-37). Finally, for *GOB* (ORF 2054) we observed a fold change decrease of -2.2 and changed the mRNA count from 248 to 112 (-136). When examining all these relevant proteins it can be seen that there is a total mRNA count decrease -435. However when examining only the total mRNA change between *blab*, *CME*, and *GOB* there was a total decrease of -118 of known beta-lactamases.

Under imipenem conditions there was relevant positive fold change at ORF 605, 666, 1724, *GOB* and 2959. For ORF 605 we observed a fold change increase of +1.7 and changed the mRNA count from 471 to 797 (+326). For ORF 666 we observed a fold change increase of +2.5 and changed the mRNA count from 930 to 2332 (+1402). For ORF 1724 we observed a fold change increase of +2.5 and changed the mRNA count from 14 to 35 (+21). For *GOB* (ORF 2054) we observed a fold change increase of +1.6 and changed the mRNA count from 249 to 397

(+149). Finally, for ORF 2959 we observed a fold change increase of +2.4 and changed the mRNA count from 5 to 12 (+7). There were also relevant fold change decreases at ORF 503(*CME*) and ORF 1103. For *CME* we observed a fold change decrease of -2.2 and changed the mRNA count from 71 to 32 (-39). Then for ORF 1103 we observed a fold change decrease of -1.5 and changed the mRNA count from 63 to 42 (-21). The combination of the up and down regulated genes leads to a total mRNA change of +1821, with the majority of the changes coming from ORF 605 and ORF 666. All fold change for all beta-lactamases can be seen in figure 4.

Table 3: mRNA fold change chart for Beta-lactamase production with operons. mRNA counts too low for relevance are in blue, fold change increases are in green, and fold change decreases are in orange

ORF	Gene Description	mRNA Count Control	mRNA Counts Cefotax	mRNA Counts Imipenem	Fold Change Cefotax	Fold Change Imipenem	Operonic?
29	Beta-lactamase (EC 3.5.2.6)	7	6	9	-1.2	1.3	None
194	Beta-lactamase (EC 3.5.2.6)	116	132	109	1.1	-1.1	None
339	Beta-lactamase (EC 3.5.2.6)	1	1	1	1	1	338, 339
503	Beta-lactamase (<i>CME</i>)	71	56	32	-1.3	-2.2	502, 503, 504, 505
519	Beta-lactamase (EC 3.5.2.6)	48	19	37	-2.5	-1.3	519, 520, 521
605	Beta-lactamase (EC 3.5.2.6)	471	797	685	1.7	1.5	None
666	beta-lactamase domain protein	930	242	2332	-3.8	2.5	666, 667
1010	Beta-lactamase (<i>Putative TLA</i>)	71	34	72	-2.1	1	None
1103	beta-lactamase domain protein	63	106	42	1.7	-1.5	1101, 1102, 1103
1724	Beta-lactamase (EC 3.5.2.6)	14	12	35	-1.2	2.5	None
1915	Beta-lactamase (<i>blaB</i>)	89	144	69	1.5	-1.3	None
1990	Metallo-beta-lactamase superfamily protein PA0057	1	3	3	3	3	1988, 1989, 1990
2054	Metalo Beta-Lactamase precursor (<i>GOB</i>)	248	112	397	-2.2	1.6	2052, 2053, 2054, 2055, 2556, 2557, 2558, 2559, 2560
2536	Beta-lactamase	16	17	12	1.1	-1.3	None
2959	AmpG protein, beta-lactamase induction signal transducer	5	6	12	1.2	2.4	2959, 2960
3527	Beta-lactamase (EC 3.5.2.6)	24	19	21	-1.2	-1.1	3527, 3528

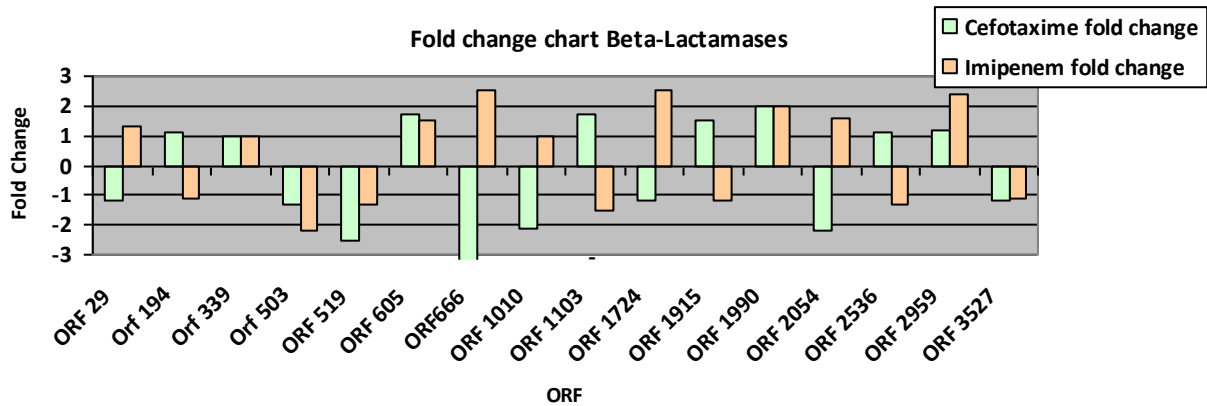


Figure 4: Fold change graph Beta-lactamases. X-Axis is ORF number Y-Axis is fold change

Discussion

Elizabethkingia is an important emerging pathogen whose mechanisms for antibiotic resistance are not well characterized. In order to test the hypothesis of there being unique regulation of genes under different antibiotic conditions we examined the fold change in relevant gene expression in both the cefotaxime and imipenem cultures. With the information gathered from this experiment it appears to support the hypothesis that *E. anopheles* expresses distinct responses to each antibiotic, and we were able to identify ORF666, a putative MBL as potentially playing an important role in resistance to imipenem.

In the peptidoglycan synthesis pathway under cefotaxime conditions the production of the putative D-alanyl-D-alanine carboxypeptidase protein we observed a down regulation of two ORFs (187 and 646) and an up regulation of one ORF (1250). This regulation led to an overall mRNA count increase of +156 with ORF 1250 having a mRNA increase of +285. This total increase could be relevant because it may imply that the cell requires greater peptidoglycan synthesis. This could be due to beta-lactamases not disrupting all beta-lactams and causing some to enter the cell, causing some PBP to be made inactive. If PBP were inhibited the cell would need to increase synthesis of PBP in order to compensate for the loss. It could also imply that the

cell is switching to more efficient peptidoglycan synthesis mechanism that could have decreased binding affinity to cefotaxime. It is also possible that these changes in regulation could be due to the cell having differing growth that causes the cell progresses slower or faster through the growth cycle. However, without further testing it cannot be known why this regulation occurs. But, while we may not be able to determine the reason for the up regulation at this time we can see that the response under imipenem is very different. Under imipenem there was only relevant up regulation of one ORF (995) and it lead to an overall mRNA count of +7. In comparison to the cefotaxime conditions, the mRNA change in imipenem is roughly 22 times smaller than the change under cefotaxime. This difference change may suggest that under imipenem conditions there is not a large need for increased peptidoglycan. One explanation for this could due to the production of more effective beta-lactmase functionality, allowing the cells to grow at a rate that is more comparable to the control conditions. An alternative explanation could be that the imipenem culture had its exponential phase slowed by the presence of imipenem and is still synthesizing large amounts of peptidoglycan, while the cefotaxime may not. However, the addition of the antibiotic when all cultures were at an OD₆₀₀ of 0.8 argues against this. The redundant nature of the gene could be due to a variety of reasons. One reason could be that each of these putative D-alanyl-D-alanine carboxypeptidase have different specificity or affinities that are activated by signaling pathways in the cell. This could possibly explain the difference in regulation amoung the two antibiotic enviorments. It's also possible that the redundancy is caused by the cell requiring different effective D-alanyl-D-alanine carboxypeptidase during each cell cycle, causing alternate expression at each phase of cell growth. One possible method of testing this hypothesis could be done by performing a real time PCR to determine mRNA count changes.

The production of RND efflux pump proteins we observed a similar trend of regulation to that of peptidoglycan synthesis with cefotaxime causing only up regulation and the imipenem causing a combination of up and down regulation. Under cefotaxime there was only up regulation leading to a total mRNA increase of +1,534. This increase in mRNA transcripts could suggest that the production of RND efflux pumps may be stimulated by the presence of cefotaxime. However, more research is required in order to confirm this. Also, according to the proteins name at ORF 2869 & 3436 these proteins encode for both inner and outer membrane pumps. This large increase in both inner and outer membrane pumps could suggest that cefotaxime causes the cell to up regulate pumps to remove cefotaxime from the periplasmic space. Imipenem treated cells have opposite regulation with a total mRNA count decrease of -687 and both ORF 2869 & 3436 being down regulated. This change in relevant mRNA count is interesting because when comparing cefotaxime and imipenem it can be seen that relevant gene regulation caused a difference in mRNA count by 2,212. This number is relevant and could imply that the proteins at ORF 2869 & 3436 are possibly inducible and relevant for antibiotic resistance, however *in vivo* work is needed to confirm this possibility. This change in regulation was in accordance with the information presented by Li *et al.* (1994) when they predicted that hydrophilic antibiotics would be excreted from the cell due to the increase in production of efflux pumps. The data shows that under cefotaxime conditions (a hydrophilic antibiotic)²¹ production of efflux pumps increased significantly, while under imipenem conditions (a hydrophobic antibiotic)²² efflux pump production decreased. Another important factor to consider is that both of these proteins normally express large number of mRNA counts under standard conditions. This could imply that these pumps have important physiological functions that are not related to antibiotic resistance. However, more research is required in order to confirm this.

Finally, beta-lactamase production had an overall decrease of -118 while under cefotaxime conditions. Of the known beta-lactamases *CME* did not have relevant change, *blaB* increased, and *GOB* and the putative *TLA* decreased. *BlaB* had an increased mRNA count of +43 while *GOB* had a mRNA decrease of -137. The change in these known beta-lactamases suggests that the up regulation *blaB* is due to the introduction by cefotaxime. This could be due to *blaB* being more effective at hydrolyzing cefotaxime or could simply be caused by some condition of cefotaxime inducing *blaB*. This response coincides with research Boschi (2000) that found that there was a putative correlation between cephalosporins and *blaB*.²³ Also, although is not characterized it did have a *TLA* relevant mRNA decrease of -37. Besides from *TLA* and the known beta-lactamases a putative beta-lactamase at ORF 666 had relevant mRNA decrease of -688. This mRNA change is the greatest out of all of the genes in the beta-lactamase chart and could be a beta-lactamase or a protein important to beta-lactamases due to its large mRNA changes. Under imipenem conditions *GOB* is upregulated and *CME* is down regulated. This increase in *GOB* is possibly induced by an environment factor created from imipenem. For all genes under imipenem there was a total relevant mRNA increase of +1821. There was also a relevant increase once again at ORF 666 where it had a total mRNA change of +1402. This mRNA change is extremely large in comparison to all other genes in this pathway. It possible that this putative protein may have some relation to *GOB* since its regulation under each antibiotic mimicked the direction of *GOB* in both cases. Sequence identity suggests that the protein is a putative metallo beta-lactamase. If this protein actually encodes for a beta-lactamase the up regulation with *GOB* and ORF 666 could imply that some cellular condition created by imipenem could cause selection for metallo beta-lactamases

Conclusions and future directions

The data suggests that *E.anophelis* uses different mechanisms when interacting with specific antibiotics. This is because when examining relevant fold change genes there are drastic differences. In the peptidoglycan synthesis pathway cefotaxime exposed cells had a 22 fold greater up regulation compared to imipenem exposed cells. In the RND efflux pump pathway there was a difference of 2212 mRNA count change between relevant genes. Finally, beta-lactamase seemed to be produced in greater numbers when exposed to cefotaxime and imipenem. *GOB* specifically seemed to be up regulated by the presence of imipenem but down regulated under cefotaxime. This in conjunction with the putative beta-lactamase at ORF 666 lead to a 2000+ mRNA count change among beta-lactamases. When reacting with cefotaxime the data suggests that *E. anophelis* may have increased production of RND efflux pumps and peptidoglycan synthesis products. While significantly decreasing the production of beta-lactamases except BlaB. However, when reacting to imipenem it is likely that *Elizabethkingia anophelis* responds with a down regulation of RND efflux pump but increases the production peptidoglycan synthesis products and begins to produce greater number of *GOB* beta-lactamases and an unknown putative metallo beta-lactamase at ORF 666.

More research is required to test the change in response to beta-lactam antibiotics. I would recommend attempting to grow *Elizabethkingia anophelis* R26 cultures that have relevant genes knocked out. This would help to show the significance or insignificance of the gene under each condition. The first gene to look at would be the putative metallo-beta-lactamase at ORF 666. This change in resistance could be tested by transforming this putative metallo-beta-lactamase into *E. coli* or another bacterial species. Then observe if bacteria were able to grow under

imipenem conditions and not under cefotaxime conditions. If the bacteria have specified resistance it would suggest ORF 666 is a novel and functional metallo-beta-lactamase.

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